Pergamon

Bioorganic & Medicinal Chemistry 6 (1998) 1379–1388

BIOORGANIC & MEDICINAL CHEMISTRY

Synthesis, Biological Activity, and Conformational Studies of Insect Allatostatin Neuropeptide Analogues Incorporating Turn-Promoting Moieties†

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Received 13 March 1998; accepted 25 June 1998

Abstract—Allatostatins are 6–18 amino acid peptides synthezed by insects to control production of juvenile hormones, which in turn regulate functions including metamorphosis and egg production. Four insect allatostatin neuropeptide analogues incorporating turn-promoting pseudopeptide moieties in the region responsible for biological activity were prepared by solid phase peptide synthetic methods. Bioassay indicated that activities approached those of the natural neuropeptides, and molecular models based on NMR data showed similar conformations and the presence of a β-turn in the active core region for the four analogues. Differences in activity are believed to be due to differences in bulk and relative position of atoms in the unnatural portion of the analogues, and their differing degrees of conformational freedom. The studies support the feasibility of development of neuropeptide-based insect control agents resistant to peptidase deactivation.

Introduction

Insect neuropeptides control essential physiological processes such as maturation, water balance, pheromone production, and metamorphosis.[1,2] Natural insect neuropeptides are readily degraded by peptidases; however, neuropeptide analogues which retain activity but resist peptidase degradation are attractive leads in the development of environmentally safe insect control agents.[3] Preparation of such analogues requires knowledge of the chemical and conformational features of the neuropeptides necessary for activity. Allatostatins, members of a family of 6–18 amino acid peptides originally isolated from the cockroach *Diploptera punctata*,[4–7] which inhibit juvenile hormone (JH) synthesis and thereby control insect maturation and egg production, were chosen as targets. Earlier structure–activity studies have shown that the carboxyl region pentapeptide Tyr-Xaa-Phe-Gly-Leu-NH_2 (Xaa = Ala, Asn, Gly, Ser) was shared in all identified neuropeptides, and was the minimum sequence capable of eliciting inhibition of JH production.[8] The amino terminal sequence, on the other hand, was varied in length and composition, indicating the importance of the carboxyl terminal region for binding with the receptors.

Successful interaction of the allatostatins with the receptor site requires that an 'active conformation' be adopted by the core region during the process of binding and activation. Secondary structure predictions and energy minimizations of the linear allatostatin pentapeptide suggested that a turn in the active core over residues Xaa-Phe-Gly-Leu could represent a plausible active conformation.[8] Similar conformational motifs within the active core sequences have been identified in insect neuropeptides of the pyrokinin/PBAN, insect

Key words: Allatostatins; β-turn; insect neuropeptides; molecular modeling; NMR.

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†Dedicated to Professor Stuart Schreiber on the occasion of his award of the Tetrahedron Prize.

0968-0896/98/$—see front matter
PII: S0968-0896(98)00129-1

kinin, and myosuppressin families.[9–11] In order to prove the existence of a turn in the active conformation, to determine its importance in binding and biological activity, and to study the possibility of preparing insect control agents which were neuropeptide-like but not readily susceptible to peptidase inactivation, we designed a series of allatostatin analogues containing unnatural moieties within the carboxyl terminal region which would be expected to promote turn formation. We here report their synthesis, biological activity, and conformational analysis as determined by NMR spectroscopy and molecular modeling.

Results and Discussion

Analogue design and synthesis

In order to provide definitive experimental evidence for a turn as the active allatostatin conformation, four mimetic analogues were designed and synthesized incorporating restricted conformation components as replacements for the 'corner' or 'pivot' residues Phe (i + 1) and Gly (i + 2) within the putative four-residue turn. In the first, the Phe residue was replaced with an Aic (aminoindane carboxylic acid) residue, containing an indane ring system. The α,α-disubstituted residue, with a cyclopentyl ring incorporating the α-carbon, was expected to promote the formation of a turn in the backbone of this region of the sequence,[12] while at the same time allowing for retention of the phenyl sidechain ring critical for biological activity (Fig. 1, **1**).[8] In the second, the Gly residue was replaced with an even more restricted residue, Cpa (cyclopropylalanine), containing a cyclopropyl ring incorporating the α-carbon (Fig. 1, **2**).

The last two analogues, also shown in Figure 1, were designed to replace both corner residues with conformationally-restricted moieties. In analogue **3**, both the indane ring moiety Aic and the cyclopropyl ring moiety Cpa were incorporated in the same structure as replacements for the pivot residues. In analogue **4**, residues i + 1 and i + 2 were replaced with a [1,4]-benzodiazepine turn mimic system,[13] specifically designed to promote a turn over the region occupied naturally by the four-residue block Asn-Phe-Gly-Leu in the natural allatostatins. An attractive feature of the [1,4]-benzodiazepine moiety is that it projects a phenyl ring in the same region as would be occupied by the critical phenyl ring of the sidechain of the native Phe residue. As racemic (*R,S*) Fmoc-3-amino-*N*-1-carboxymethyl-2-oxo-5-phenyl-1,4-benzodiazepine was utilized in the synthesis, two pseudopeptide diastereomers with divergent biological activity were isolated as products.

1: Ala-Arg-Pro-Tyr-Asn-Aic-Gly-Leu-NH_2
2: Ala-Arg-Pro-Tyr-Asn-Phe-Cpa-Leu-NH_2
3: Ala-Arg-Pro-Tyr-Asn-Aic-Cpa-Leu-NH_2
4a,b: Ala-Arg-Pro-Tyr-Asn-Bzd-Leu-NH_2

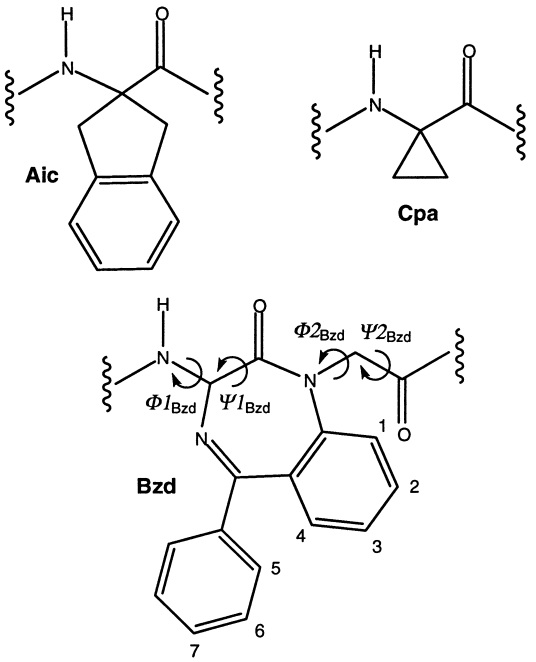

Figure 1. Allatostatin analogues **1–4b** amino acid sequence and chemical structure of the unnatural residues Aic, Cpa, and Bzd. The numbering used for the aromatic protons in Bzd is indicated.

Synthesis of the unnatural peptide analogues was carried out using Fmoc chemistry, primarily with the coupling reagent mixture of one equiv each of 1,3-diisopropylcarbodiimide/1-hydroxy-7-azabenzotriazole (HOAt) in dimethylsulfoxide. However, coupling of the sterically hindered α,α-disubstituted amino acids containing indane and cyclopropyl ring systems to the peptide resin complex, and coupling of the immediate residue thereafter was effected with more stringent conditions, consisting of one equiv of [*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] (HATU) with two equiv *N,N*-diisopropylethylamine in dimethylsulfoxide for a longer period. The resin utilized with peptide analogues containing the α,α-disubstituted amino acids was methylbenzhydrylamine (MBHA), normally used in *t*-BOC peptide synthesis strategies, due to its apparently greater stability to exposure to the HATU coupling reagent than Rink Amide resin.

Biological assays

The allatostatin analogues were evaluated for their ability to inhibit biosynthesis of JH by the corpora

allata (CA) of the cockroach *D. punctata*, maintained in vitro, by measuring the incorporation of radiolabelled precursors. Despite the major conformational and steric constrictions imposed on the allatostatin sequence by turn-promoting moieties in these analogues, all demonstrated significant biological activity and complete retention of efficacy within the physiological range of concentrations, as summarized in Table 1 and seen in a representative dose–response curve shown in Figure 2. Indeed, the activity of the indane analogue **1** proved roughly equivalent to that of the natural allatostatin peptide it mimicked, with an ED_{50} of 3.2 nM. By comparison, the Dip-AST-6 peptide demonstrates an ED_{50} of 2.3 nM. Analogue **2**, containing the more constricted cyclopropyl ring moiety, was less active but still demonstrated significant activity with an ED_{50} of 0.2 μM. Analogue **3**, containing both the indane and cyclopropyl ring moieties, also retained activity within the physiological range with an ED_{50} of 0.8 μM. Compound **4a**, containing the [1,4]-benzodiazepine moiety, proved to have allatostatin-like inhibition of JH-biosynthesis with an ED_{50} of 10 μM, whereas its diastereomer **4b** demonstrated apparent stimulation of JH biosynthesis between 0.1 and 10 μM. The analogue at 10 μM was found to partially antagonize effects of a 10 nM solution of the natural allatostatin Dip-AST-5. It was postulated that such a response could have resulted from an ability of the analogue to bind, but not activate, the allatostatin receptor which thereby prevented endogenous allatostatin in the bioassay system from reaching the receptor site.

NMR studies

Spectral assignments of normal amino acids were made for the most part by comparison of chemical shifts with literature values,[14] with signals from Tyr and Phe

Table 1. Inhibition of JH release by corpora allata of *Diploptera punctata*

Analogue	ED_{50}	Maximal responses[a]
Dip-AST-6	2.30 nM	90%
1	3.20 nM	87%
2	0.16 μM	93%
3	0.80 μM	92%
4a	10.0 μM	90%
4b	[−15% (Stimulatory) (0.1–10 μM)][b]	

[a]ED_{50} values determined from dose–response curves whose data points represent means of eight replicates. Maximal response is not statistically significantly different from natural analogues Dip-AST-5 or Dip-AST-6. Data represents % of inhibition of JH release by CA of *Diploptera punctata*.

[b]Analogue **4b** at 10 μM partially antagonized the JH biosynthesis inhibitory properties of a 10 nM solution of Dip-AST-5.

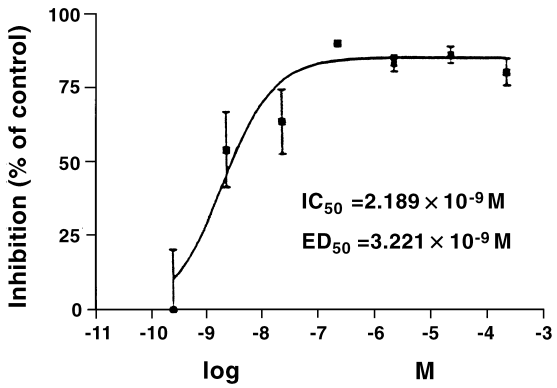

Figure 2. Dose–response curve for inhibition of JH release by the corpora allata of the cockroach *Diploptera punctata* by restricted-conformation analogue **1** (Fig. 1). Data points are means of eight replications and the vertical lines through each point represents the standard error.

assigned using NOE correlations to their respective aromatic protons. For the pseudopeptide Aic in analogues **1** and **3**, the N-H appeared as a singlet at 8.46 and 8.40 ppm, respectively, and the four cyclopentyl ring protons appeared as doublets near 3.50 ppm. The identity of the pair of doublets produced by protons on the face nearer the amide could be determined by NOE correlations to the amide proton. Cpa also produced a singlet N-H at 8.53 and 8.51 ppm in analogues **2** and **3**, respectively, and the four ring protons produced broad pairs of peaks at high field. These could also be assigned using amide NOE correlations. For analogue **3**, containing both moieties, back NOE correlations from the ring protons were used to determine the N-H peak identities. For the [1,4]-benzodiazepine pseudopeptide in analogue **4a**, the amide proton produced a doublet at 8.74 ppm coupled to the α-hydrogen doublet at 5.43. Proton chemical shifts for the four analogues are recorded in Table 2.

Data useful for modeling included temperature gradients of 4.0, 4.2, and 4.5 ppb/°C for the Leu amide proton in analogues **1, 2**, and **3**, respectively, evidence for the presence of hydrogen bonds, and a coupling constant $^3J_{HN\alpha}$ of 3.5 Hz for the Phe residue in analogue **2**. Important NOE correlations for analogue **1** included a strong interaction between Asn Hα and the Aic N-H, medium interactions between the Gly N-H and Leu N-H, and weak interactions between Aic N-H and Gly N-H, and Gly Hα and Leu N-H. For analogue **2**, a strong interaction was observed for the Phe Hα to Cpa N-H, medium interactions were between Asn Hα and Phe N-H, Asn Hα and Asn N-H, and Phe N-H and Cpa N-H. In analogue **3**, medium interactions were observed for the Asn Hα and Aic N-H, Cpa N-H and Leu N-H, and

Table 2. NMR assignments for allatostatin analogues (chemical shifts are indicated in ppm)

Analogue	Residue	Hα	Hβ	N–H	Other
1	Ala	4.09	1.50	—	—
	Arg	4.61	1.70	8.78	1.70 Hγ
					3.17 Hδ
					7.23 NHε
	Pro	4.36	1.66, 2.24	—	1.83, 1.92 Hγ
					3.04, 3.81 Hδ
	Tyr	4.39	2.85, 3.00	8.60	7.12 Hγ
					6.82 Hδ
	Asn	4.40	2.49, 2.76	8.50	6.86, 7.64 NH_2
	Aic[a]	—	3.33, 3.64 3.15, 3.58	8.46	7.26 H_{arom}
	Gly	3.83, 3.92	—	8.29	—
	Leu	4.30	1.61	7.87	1.75 Hγ
					0.85, 0.93 Hδ
					7.20, 7.64 NH_2
2	Ala	4.08	1.51	—	—
	Arg	4.59	1.69	8.62	1.58 Hγ
					2.96 Hδ
					7.01 NHε
	Pro	4.46	1.92, 2.34	—	2.05 Hγ
					3.65, 3.84 Hδ
	Tyr	4.42	2.95, 3.05	8.47	7.11 Hγ
					6.83 Hδ
	Asn	4.51	2.68	8.13	6.80, 7.52 NH_2
	Phe	4.18	2.91, 3.15	7.86	7.38 Hγ
					7.21 Hδ
					7.34 Hε
	Cpa[b]		1.22, 1.36, 0.55, 0.66	8.53	—
	Leu	4.27	1.53, 1.60	7.54	1.64 Hγ
					0.82, 0.88 Hδ
					7.03, 7.52 NH_2
3	Ala	4.09	1.50	—	—
	Arg	4.58	1.71, 1.66	8.45	1.63, 1.65 Hγ
					3.13 Hδ
	Pro	4.33	1.68, 2.22	—	1.83, 1.92 Hγ
					3.04, 3.81 Hδ
	Tyr	4.37	2.95, 3.86	8.41	7.11 Hγ
					6.82 Hδ
	Asn	4.44	2.56, 2.81	8.35	6.83, 7.54 NH_2
	Aic[a]	—	3.28, 3.62 3.11, 3.55	8.40	7.25 H_{arom}
	Cpa[b]	—	1.04, 1.10 1.45, 1.49	8.51	—
	Leu	4.29	1.62, 1.64	7.71	1.79 Hγ
					0.86, 0.96 Hδ
					7.10, 7.48 NH_2
4a	Ala	4.06	1.47	—	—
	Arg	4.61	1.69, 1.77	8.59	1.62 Hγ
					3.13 Hδ
					7.08 NHε
	Pro	4.37	1.93, 2.20	—	1.84, 1.93 Hγ
					3.56, 3.75 Hδ
	Tyr	4.51	3.01	8.21	7.09 Hγ
					6.72 Hδ
	Asn	—	2.67, 2.78	8.36	6.86, 7.57 NH_2
	Bzd[c]	5.43	—	8.74	7.36 H_6
					7.45 H_1, H_4
					7.54 H_2, H_3, H_7
					7.71 H_5
	Leu	4.21	1.54	8.48	1.54 Hγ
					0.86, 0.79 Hδ
					6.95, 7.42 NH_2

[a,b]For Aic and Cpa protons on the same Cβ are listed contiguously. The assignment of the different β-proton pairs is described in the text.
[c]The numbering for the aromatic protons in Bzd is shown in Figure 1.

Leu N-H and Leu Hα, and weak interactions between the Asn N-H and Asn Hα, and the Aic N-H and Cpa N-H. Finally, for agonist analogue **4a**, medium interactions were between Asn Hα and Bzd N-H, and Bzd N-H and Bzd Hα, and weak interactions between Asn N-H and Bzd N-H, and Leu N-H and Leu Hα.

Conformational studies

As discussed in the previous section, the NOE correlations found for the residues in the active core region and the small temperature gradients found for the amide protons of the Leu residue in three of the analogues indicate that the presence of a turn is likely. For analogue **2** this is also evidenced by a $^3J_{HN\alpha}$ coupling constant of 3.5 Hz for Phe, characteristic of a β-turn of type I or II.[15] However, weak correlations or lack of them makes the determination of unique conformations impossible from NOE data alone. The use of amide temperature gradients as structural constraints is usually permissible only if the carbonyl acceptor can be identified. Incorrect assignment of the acceptor atom can lead to severe distortions in the resulting structures and misinterpretation of the molecular modeling results. To avoid any ambiguities when including this type of information, and because only one residue of the active core region shows small amide proton temperature gradients, a systematic search of all possible hydrogen bond acceptors was carried out for analogues **1, 2**, and **3**. Hydrogen bonds from the amide proton of Leu-8 to the backbone carbonyl oxygen at positions 6, 5, and 4, which would be found in γ-, β-, and 3_{10}-turns respectively, and to the side chain carbonyl oxygen of Asn-5 were treated as distance range constraints with an upper limit of 2.5 Å in separate experiments. Acceptors further than four residues away were not considered since folded conformations of this size are rarely seen in short peptides,[16] and the presence of these motifs would very likely be evidenced in the ROESY spectra. A thorough conformational search was performed, resulting in sets of 200 structures for each of the possible hydrogen bonding topologies. The mean energies from the 20 lowest energy conformers from each search were compared, and the results for the three analogues are presented in Table 3. The differences in relative energies for the different hydrogen bonding topologies indicates that a β-turn is the most probable conformation for the active core region of the analogues analyzed by this protocol. Many of the low energy conformations found for the other three possible hydrogen bonding topologies also displayed a β-turn involving the Leu amide proton and the backbone carbonyl of Asn, indicating that this turn motif is preferred in these molecules. γ-Turn motifs also seem to be favored, but in vacuo simulations are known to stabilize them unrealistically and they are thus less likely.[17] The different β-turn types obtained for the three analogues and their characteristics are discussed below.

Table 3. Relative mean energies (Kcal/mol) for the different hydrogen bonding topologies in Cpa and Aic containing allatostatin analogues with respect to their β-turn conformation mean energy

Analogue	γ-turn	3_{10}-turn	Asn-5 carbonyl[a]
1	1.3	2.2	2.8
2	0.2	7.3	3.6
3	0.9	2.3	2.2

[a]Conformation involving a hydrogen bond between the Leu-8 amide proton and the side-chain carbonyl of Asn-5.

A different problem was encountered in the conformational study of analogue **4a**. Its preparation relied on racemic Bzd, and yielded two compounds with epimeric stereochemistry at the Cα carbon of this residue. While this analogue showed agonistic allatostatin activity, its epimer **4b** did not. Since the Phe residue with the natural L configuration is a requirement for activity in Dip-AST-5,[8] the strereochemistry of the equivalent chiral center in Bzd must correspond to the L configuration, which in this case is *S*. As a further test, both stereochemistries were considered in separate modeling experiments. Four hundred structures consistent with the NMR constraints derived for analogue **4a** were obtained in each case. All low energy structures modeled with the *R* stereochemistry presented a tight γ-turn involving the Leu N-H and the Bzd residue carbonyl. There is no experimental evidence for this turn, because the temperature gradient for the Leu amide proton is almost 12 ppb/°C. On the other hand, low energy structures for the *S* enantiomer were consistent with experimental data and showed no hydrogen bonding or γ-turn formation.

Superpositions of the 10 lowest energy structures found for the four analogues are shown in Figure 3, and statistics for the conformer sets are summarized in Table 4. The different β-turn conformations were assigned by comparing the average Φ and Ψ angles of residues 2 and 3 of the turn from the lowest energy structures against standard β-turn angles, allowing for a ±30° deviation from standard values as reported by Willmot and Thornton.[18] Of the three analogues containing the unnatural amino acids Aic and Cpa, **2** showed only type II β-turns, with $\Phi_{Phe} = -52°$, $\Psi_{Phe} = 130°$, $\Phi_{Cpa} = 62°$, and $\Psi_{Cpa} = -10°$. On the other hand, type I′ and II′ β-turns were found for analogues **1** and **3**. Analogue **1** presented low energy conformers with $\Phi_{Aic} = 46°$, $\Psi_{Aic} = 42°$, $\Phi_{Gly} = 73°$, and $\Psi_{Gly} = -24°$, and $\Phi_{Aic} = 57°$, $\Psi_{Aic} = -104°$, $\Phi_{Gly} = -112°$, and $\Psi_{Gly} = -9°$ for type I′ and II′ β-turns, respectively. For analogue **3**, the type I′

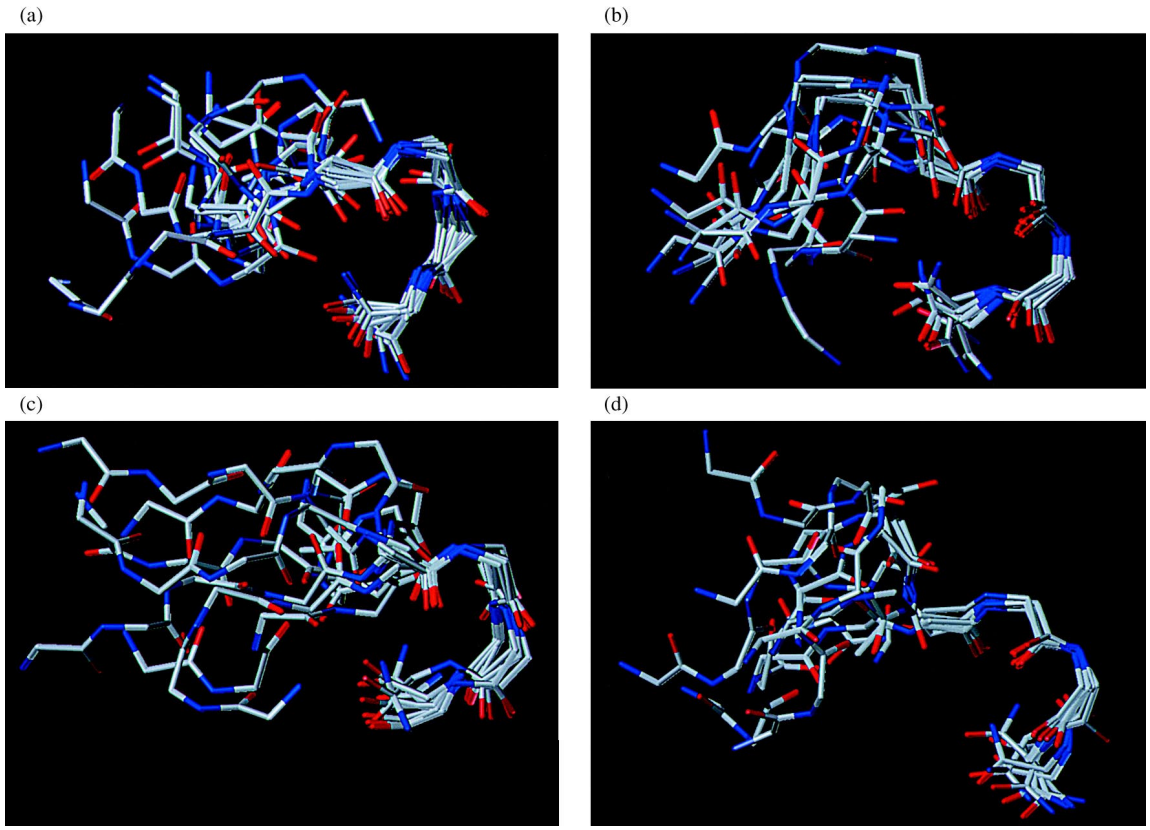

Figure 3. Backbone atom superposition of the low energy structures obtained for analogues **1** (a), **2** (b), **3** (c), and **4a** (d). Only backbone atoms are shown for clarity. See text for details and Table 4 for fit statistics.

and II′ β-turns had $\Phi A_{ic} = 42°$, $\Psi_{Aic} = 50°$, $\Phi_{Cpa} = 67°$, and $\Psi_{Cpa} = -15°$, and $\Phi_{Aic} = 46°$, $\Psi_{Aic} = -130°$, $\Phi_{Cpa} = -57°$, and $\Psi_{Cpa} = -18°$, respectively. The presence of inverse turns in these two analogues is consistent with reported findings for structures containing α,α-disubstituted unnatural amino acids as pivots in position 2 of β-turns.[12] Finally, analogue **4a** indicated only the presence of an open turn in the active core region, with $\Phi 1_{Bzd} = -69°$, $\Psi 1_{Bzd} = 165°$, $\Phi 2_{Bzd} = 67°$, and $\Psi 2_{Bzd} = -15°$, which can be classified as a type II β-turn.

Table 4. Statistics for the low energy conformations of allatostatin analogues shown in Figures 2 and 3 (RMS deviations are for the superposition of active core region residues)

	All atoms		Backbone atoms	
Analogue	RMSD (Å)	SD	RMSD (Å)	SD
1	1.2	0.2	0.41	0.08
2	1.7	0.2	0.50	0.12
3	1.9	0.5	0.77	0.20
4a	1.4	0.3	0.52	0.08
All[a]	—	—	0.80	0.40

[a]RMSD between lowest energy structure of the four analogues as shown in Figure 4.

Overall, the proposed structure of the active core region is similar in the four analogues studied. Despite their different chemical structure, a superposition of the backbone atoms of this region, shown in Figure 4, has an RMSD of only 0.8 Å. High conformational freedom is also observed for the Ala, Arg and Pro residues in all the analogues, which is consistent with the lack of NOE interactions, high temperature gradients for amide protons, and rotationally averaged values for $^3J_{HH}$ and $^3J_{HN\alpha}$ coupling constant observed for these residues.

Conclusions

As shown in Table 1, activity of the neuropeptide analogues prepared with unnatural amino acids and peptidomimetics approached and, in the case of analogue **1**, virtually matched that of the natural neuropeptide, indicating that presence of these turn promoting moieties in the portion of the molecule necessary for activity

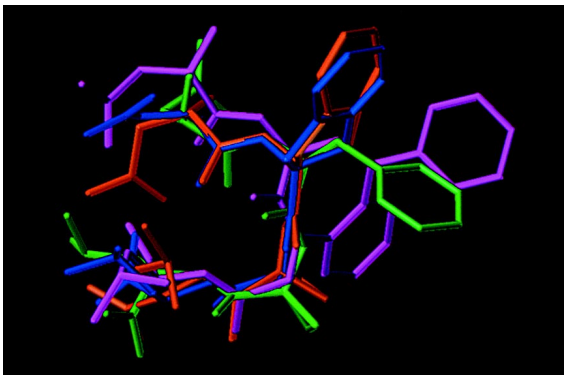

Figure 4. Backbone atom superposition of the active core region residues of the four different allatostatin analogues. Hydrogen atoms omitted for clarity.

did not eliminate potency or adversely affect efficacy. The low temperature gradient of the Leu amide proton found in three compounds, consistent with hydrogen bond formation, and the $^{3}J_{HN\alpha}$ coupling constant of 3.5 Hz for the Phe residue in analogue **2** indicated that turns were present in the molecules. Comparison of relative energy levels for possible turn types indicated that β-turns over the region occupied in the natural peptide by the carboxyl terminal residues Asn-Phe-Gly-Leu were favored in each case, as shown in Table 3. Superposition showed similar conformations for backbones of all four analogues, with only slight differences in position of the fixed side chains which could easily account for the differences in biological activity observed. For example, the somewhat open turn conformation and higher backbone rigidity found for analogue **4a** could explain its weaker biological activity. The high activity exhibited by analogue **1** and the rigidity imposed on its sidechain conformation by the cyclopentyl ring also indicate that the receptor site can accomodate a sidechain phenyl ring perpendicular to the neuropeptide backbone. While other turn types were not greatly higher in energy for at least two of the analogues, and active site interaction does not necessarily involve the lowest energy form, the alternate turns are not compatible with NMR and molecular modeling data obtained on the remaining two. The close fit of the low energy β-turn structures of the four analogues is strong evidence that the β-turn is present in the natural compounds during receptor interaction.

As these conformationally restricted analogues necessarily involve incorporation of sterically hindered molecular structures, they have potential for enhanced resistance to degradation by endopeptidases present in the hemolymph (blood) or tissues of insect targets. The analogues produced for this study have this characteristic, and, although prepared as model compounds to determine active conformation, are now undergoing evaluation of their peptidase resistance and insect control properties. In view of the encouraging results of these preliminary studies, further analogues of allatostatins and other insect neuropeptide classes are now being prepared and tested.

Experimental

Peptide synthesis

The allatostatin pseudopeptide analogues were synthesized using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry on either Rink Amide (0.56 mEq/g) or methylbenzhydrylamine (MBHA) resin (0.32 mEq/g) (Novabiochem, San Diego, CA) with a Milligen/Biosearch 9600 synthesizer. Fmoc protected amino acids were purchased from Advanced Chemtech (Louisville, KY), with the protected analogues Fmoc-Tyr(OtBu)-OH and Fmoc-Arg(Pmc)-OH used for these amino acids containing sidechain functional groups. 1-Amino-1-cyclopropanecarboxylic acid (Aldrich Chemical Co., Milwakee, WI) was protected with an Fmoc group using the reagent Fmoc-*N*-hydroxysuccinamide under general conditions as previously described.[19] Fmoc-2 amino-indane-2-carboxylic acid (Fmoc-Aic) and (*R,S*)-Fmoc-3-amino-*N*-1-carboxymethyl-2-oxo 5-phenyl-1,4-benzodiazepine were purchased from Neosystem Laboratoire (Strasbourg, France). Coupling reagents used for the majority of amino acid condensations were one equiv each of 1,3-diisopropylcarbodiimide/1-hydroxy-7-azabenzotriazole (HOAt) in dimethylsulfoxide for 1 h as described earlier.[20–22] However, coupling of the α,α-disubstituted amino acids containing indane and cyclopropyl ring systems and the immediately following residue to the peptide-MBHA resin complex was effected with the more stringent reagent, one equiv of [*O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] (HATU) (PerSeptive Biosystems, Marlborough, MA) with two equiv *N,N*-diisopropylethylamine in dimethylsulfoxide shaken for 4 h. Removal of the N-terminal Fmoc groups from the α,α-disubstituted amino acids was accomplished with 20% piperidine in dichloromethane for 1 h rather than the 30 min used for the other residues. The product was cleaved from Rink Amide resin by stirring the peptide–resin complex with a mixture of trifluoroacetic acid (90%)/anisole (5%)/thioanisole (4%)/1,2-ethanedithiol (1%) for 1.5 h at ambient temperature. The resin suspension was filtered and volatile reagents were removed in vacuo on a Savant (Farmingdale, NY) Speed Vac concentrator at 40 °C. Peptide–resin complexes made with the MBHA resin were cleaved with anhydrous hydrogen fluoride (HF) (10 mL/g resin) in the presence of excess anisole (1.5 mL/g resin) and 1,2-ethanedithiol

(0.5 mL/g resin) for 1 h at 0 °C. Anisole and ethanedithiol were removed by a diethyl ether extraction. Crude peptide samples were purified on a Waters C18 cartridge and a Delta Pak C18 reverse-phase column on a Waters 510 HPLC controlled with a Millenium 2010 chromatography manager system (Waters, Milford, MA) with detection at 214 nm at room temperature. Solvent A = 0.1% aqueous trifluoroacetic acid (TFA); solvent B = 80% aqueous acetonitrile containing 0.1% TFA. Conditions: Initial solvent consisting of 20% B was followed by Waters linear program 6-1005B over 40 min; flow rate 2 mL/min. Retention times: **1**, 18.5 min; **2**, 16.5 min; **3**, 19.5 min; **4a**, 23.0 min; and **4b**, 22.2 min. Analogue **3** was further purified on a Waters Protein Pak 125 column. Sovent A = 95% aqueous acetonitrile containing 0.01% TFA, solvent B = 50% acetonitrile containing 0.01% TFA. Conditions: 100% A isocratic for 4 min followed by linear program to 100% B over 8 min. Retention time: **3**, 24.3 min. The pure peptides were analyzed and quantitated via amino acid analysis. Each peptide sample was purged with N_2 and the peptide hydrolyzed with vapor-phase HCl for 24 h at 105 °C. Precolumn derivatization and HPLC analysis was accomplished by the standard PicoTag® method supplied by Waters (Milford, MA). The observed amino acid ratios were as expected for each analogue: **1**, A(1.0), G(1.2), L(1.0), N(1.0), P(1.0), R(1.0) and Y(0.8); **2**: A(1.0), F(1.0), L(1.0), N(1.0), P(1.0), R(0.9) and Y(1.0); **3**: A(1.2), L(1.0), N(1.0), P(1.1), R(1.1) and Y(0.9); **4a**: A(1.2), L(1.0), N(1.2), R(1.1), P(1.2) and Y(0.9); **4b**: A(1.2), L(1.0), N(1.0), R(1.1), and Y(0.9). Fast atom bombardment (FAB) mass spectra were obtained by adding 10 μg of analogue sample to glycerol (1.5 μL) on a copper probe, followed by bombardment with 8 kV Xe atoms on a Kratos MS-50 mass spectrometer (Kratos, Manchester, UK).[22] The structural identity and a measure of purity of the analogues were confirmed by the presence of the following molecular ions (MH+) and absence of contaminant peaks: **1**: 948.6 [calcd MH^+: 948.51]; **2**, 962.7 [calcd MH^+: 962.52]; **3**: 974.6 [calcd H^+: 974.52]; **4a**: 1023.3 [calcd MH^+: 1023.54]; **4b**: 1023.4 [calcd MH^+: 1023.54].

Biological assays

Diloptera punctata were reared as previously described.[23] On the day of adult emergence (day 0), females were collected and maintained at 27 °C until used. Mated status was confirmed by the presence of a spermatophore and by measurement of length of basal oocytes. All radiochemical assays for JH release were performed using corpora allata (CA) from day 7 mated females. Peptide analogues were dissolved in water or in HCl (0.1 M), neutralized with NaOH (0.1 M) and added to medium 199 (GIBCO, 1.3 mM Ca^{2+}, 2% Ficoll, methionine-free) for assay as described.[4] The resulting solutions were used immediately for testing. Pseudopeptide samples were not stored long term in aqueous solution and were discarded at the end of each day. Rates of JH release were determined using the in vitro radiochemical assay,[24,25] as modified.[26,27] This assay measures the incorporation of the radiolabelled *S*-methyl moiety of radiolabelled methionine into JH III in its final step of biosynthesis by CA maintained in vitro. CA from 7 day adult mated *D. punctata* females were incubated for 3 h in 100 μL of medium 199 containing either L-[^{3}H-*S*-methyl]methionine or L-[^{14}C-*S*-methyl]-methionine (50 μM, specific radioactivity 1.48–2.03 GBq/mmol for ^{14}C-Met and 7.4 GBq/mmol for ^{3}H-Met from either New England Nuclear or Amersham). Samples were extracted and JH release determined as described.[27] Each datum point in the dose–response determinations represented replicate incubations of eight CA test pairs (i.e. allatostatin analogue added) compared to eight CA control pairs (i.e. no allatostatin analogue added).

NMR experiments

NMR spectra were acquired on a Bruker ARX-500 500 MHz spectrometer using a 5 mm HCN probe with 3-(trimethylsilyl)propionic acid, sodium salt, (TSP) as internal standard. Samples were 1–2 mM in H_2O containing 10% D_2O necessary for field-frequency lock, and water suppression was by presaturation. Spectra were acquired at 25 °C and pH 7.0 unless otherwise indicated. One dimensional spectra were recorded with 32 K data points and zero filled to 64 K, while matrix sizes of 2 K × 0.5 K data points zero filled to 2 K × 2 K were used for 2-D experiments. Linear prediction was applied prior to Fourier transformation in all experiments, and polynomial baseline correction was applied in both dimensions of the 2-D spectra. Peaks were assigned by comparison of chemical shifts with reference values and by using TOCSY and ROESY spectra when necessary. The mixing time in TOCSY experiments was 150 msec. ROESY spectra were acquired using a 250 msec hard pulse spin lock.[28] ROE correlations were classified as strong, medium, or weak depending on the volume of the 2-D spectrum cross-peaks. Temperature effects on amide ^{1}H chemical shifts were determined from the 1-D spectra acquired at 5–45 °C in 5 °C intervals.

Molecular modeling

All molecular modeling calculations were performed using Sybyl 6.3 (Tripos Associates Inc., St. Louis, MO) running on a Silicon Graphics Indigo R4000 workstation. The Weiner et al. all-atom force field (AMBER 4.0) and point charges were used for molecular dynamics and mechanics simulations,[29,30] with a distance dependent dielectric constant ($\varepsilon = R_{ij}$) to simulate

solvent effects, and an 8 Å cutoff for nonbonded interactions. Additional parameters needed to model the BZD residue with the AMBER force field were obtained as described earlier.[31] Atomic point charges for the unnatural amino acids were calculated as described by Kollman.[32] Distance ranges of 1.8–2.7 Å, 1.8–3.3 Å, and 1.8–5.0 Å were assigned for strong, medium, and weak NOE interactions, respectively. In cases were pseudoatoms were used, 0.5 Å were added to the constraint upper bound. Coupling constants from NMR experiments were included directly as constraints in the modeling experiments with Sybyl software modules developed in our laboratory.[33] A quadratic well energy penalty function with a force constant of 200 Kcal/Å^2 was employed to enforce distance range constraints, while a harmonic penalty function with a 2.0 Kcal/Hz^2 force constant was used for coupling constraints. When applicable, hydrogen bonds were treated as distance range constraints, using a force constant of 100 Kcal/Å^2 to keep the distance between hydrogen and acceptor atoms between 1.8 and 2.5 Å after energy minimization. Generation of structure ensembles was accomplished by simulated annealing experiments,[34] which consisted in 1 ps of equilibration to a thermal bath at 1000 K and exponential annealing to 200 K for a 1 ps period, followed by geometry optimization of the resulting structures to an energy gradient below 0.05 Kcal/mol. In the initial stages of the conformational search force constants in all distance range constraints were reduced to 25% of their final values to allow for a thorough search of the conformational space available to the molecules. Superposition, RMSD calculation, and comparison of structure sets and conformer families was performed with routines previously reported by us.[33,35]

Acknowledgements

The authors acknowledge the NIH for financial support (A.I.S.). We thank Jinrui Zhang, Hugo Hein and William Siefert for technical assistance. The invaluable help of Gonzalo Hernandez in the initial stages of the molecular modeling experiments is also acknowledged.

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